A PROCEDURE FOR THE MICRODETERMINATION OF CHOLINE ACETYLTRANSFERASE

Henry I. Yamamura, et al

Edgewood Arsenal
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September 1972

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by

Henry I. Yamamura, PhD
Tommy L. Gardner, BS
Alan M. Goldberg, PhD

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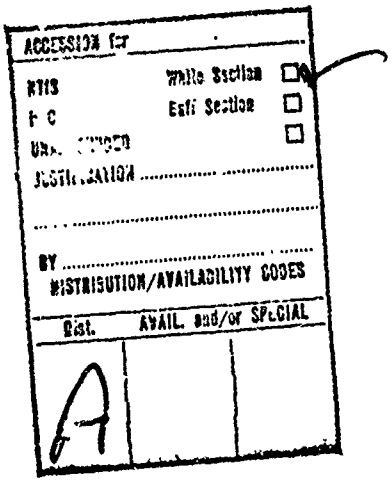

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13. ABSTRACT

A micromethod for the determination of choline acetyltransferase was developed using 300 μM acetyl Co A and 5 mM choline as substrates. The reaction yielded (^{14}C) acetylcholine, which was extracted with sodium tetraphenylboron in 3-heptanone; and an aliquot was counted utilizing a Tricarb scintillation spectrometer. The sensitivity of the method was approximately 10^{-12} moles of (^{14}C) acetylcholine synthesized/hour. Using whole rat brain homogenates, the specific activity was determined after the addition of Triton X-100 (0.5%) to the homogenate in 0.32 M sucrose. This yielded a homogenate in which choline acetyltransferase was 100% solubilized, and subsequent determinations of choline acetyltransferase yielded a relative specific activity of 35 μmoles/gram protein/hour. The removal of all brain parts caudal to the superior colliculi resulted in an increase of the specific activity to 60 μmoles/gram protein/hour This increase was due to the relatively low choline acetyltransferase activity in the cerebellum. Soman (0.1 mM) did not inhibit choline acetyltransferase activity; however, a specific tertiary inhibitor of choline acetyltransferase 4-(1-napthylvinyl)-pyridine (0.1 mM), was effective in producing 100% inhibition. A brief study was conducted using various inhibitors of choline acetyltransferase in order to determine the possibility of regulating acetylcholine synthesis and also to determine the possibility of purifying the enzyme utilizing affinity chromatography.

14. KEYWORDS

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A PROCEDURE FOR THE MICRODETERMINATION OF CHOLINE ACETYLTRANSFERASE

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Task 1W662710AD2502

DEPARTMENT OF THE ARMY
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FOREWORD

The work described in this report was authorized under Task 1W662710AD2502, Medical Defense Against Chemical Agents, Prophylaxis and Therapy for Lethal Agents. The work was started in March 1971 and completed in September 1971.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences – National Research Council.

Acknowledgments

The authors wish to express their sincere appreciation to Dr. John F. O'Leary and Mrs. Marion P. Royston for their advice and criticisms on the manuscript.

DIGEST

A micromethod for the determination of choline acetyltransferase was developed using 300 μM acetyl Co A and 5 mM choline as substrates. The reaction yielded (^{14}C) acetylcholine, which was extracted with sodium tetraphenylboron in 3-heptanone; and an aliquot was counted utilizing a Tricarb scintillation spectrometer. The sensitivity of the method was approximately 10^{-12} moles of (^{14}C) acetylcholine synthesized/hour. Using whole rat brain homogenates, the specific activity was determined after the addition of Triton X-100 (0.5%) to the homogenate in 0.32 M sucrose. This yielded a homogenate in which choline acetyltransferase was 100% solubilized, and subsequent determinations of choline acetyltransferase yielded a relative specific activity of 35 μmoles/gram protein/hour. The removal of all brain parts caudal to the superior colliculi resulted in an increase of the specific activity to 60 μmoles/gram protein/hour. This increase was due to the relatively low choline acetyltransferase activity in the cerebellum. Soman (0.1 mM) did not inhibit choline acetyltransferase activity; however, a specific tertiary inhibitor of choline acetyltransferase, 4-(1-napthylvinyl)-pyridine (0.1 mM), was effective in producing 100% inhibition. A brief study was conducted using various inhibitors of choline acetyltransferase in order to determine the possibility of regulating acetylcholine synthesis and also to determine the possibility of purifying the enzyme utilizing affinity chromatography.

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A PROCEDURE FOR THE MICRODETERMINATION OF CHOLINE ACETYLTRANSFERASE

I. INTRODUCTION.

The action of the enzyme choline acetyltransferase (or choline acetylase, acetyl Co A : choline O-acetyltransferase EC 2.3.1.6.) was first described by Korkes *et al.*[1] as the enzyme responsible for the acetylation of choline. The acetylation product of this reaction was the cholinergic neurohumoral transmitter, acetylcholine.

$$\text{Choline} + \text{Acetyl Co A} \rightleftharpoons \text{Acetylcholine} + \text{Co A}$$

The principal role of choline acetyltransferase, the biosynthesis of acetylcholine, makes it an invaluable asset as a constituent for the characterization of cholinergic function. In order to locate and identify specific cholinergic functions, many assays have been developed for the determination of acetylcholinesterase, acetylcholine, and choline; until recently, however, an efficient determination of the enzyme choline acetyltransferase with supporting kinetic data could not be obtained.[2]

Methods for the determination of choline acetyltransferase as described by Fonnum,[3] McCaman and Hunt,[4] and Shrier and Shuster[5] are relatively sensitive; but the procedures involved are complicated and prohibit the use of maximal substrate concentrations, thus limiting maximum specific activities.[4] Utilizing the nonionic solubilizer Triton X-100, as described by Fonnum,[6] and a modified extraction procedure, we were able to obtain maximum specific activities without complications. These modifications resulted in a simple and efficient method for the determination of the cholinergic indicator, choline acetyltransferase.

II. MATERIALS.

(Acetyl-1-^{14}C) Coenzyme A (50.0 mC/mmole) was purchased from Amersham/Searle, Arlington Heights, Illinois. Acetyl Coenzyme A was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Sodium tetraphenyl boron and 3-heptanone were obtained from Fisher Scientific Company, Silver Spring, Maryland. Choline chloride was purchased from Eastman Kodak Company, Rochester, New York. Triton X-100 (alkylphenoxypoly-ethoxyethanol) was obtained from Rohm and Hass Company, Philadelphia, Pennsylvania. Microcentrifuge tubes and adaptors for centrifugation were obtained from our shop facilities: (1) microcentrifuge tubes, 50 by 4 mm, and (2) test tube adaptor which was made to hold eight tubes and to fit our present centrifuge (International Refrigerated Centrifuge, Model PR-6) buckets. All micromeasurements

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[1] Korkes, S., Campillo, A., Korey, S., Stern, J., Nachmansohn, D., and Ochoa, S. J. Biol. Chem. 198, 215 (1952).

[2] Fonnum, F. Biochem. J. 109, 479 (1968).

[3] Fonnum, F. Biochem. J. 113, 391 (1969).

[4] McCaman, R., and Hunt, J. J. of Neurochem. 12, 253 (1969).

[5] Schrier, B., and Shuster, L. J. of Neurochem. 14, 977 (1967).

[6] Fonnum, F. Biochem. J. 109, 389 (1968).

were performed using H. E. Pedersen, Denmark automatic micro pipettes, 7, Sommerstedgrade, 1718 Copenhagen V Denmark.

III. METHODS.

A. Preparation of (Acetyl-1-^{14}C) Coenzyme A Substrate.

The buffer and substrate are prepared separately but are combined at the time of the assay to form the buffer-substrate solution. This method insures the stability of the substrate solution and facilitates the changing of the less stable buffer solution.

To prepare the substrate, (^{14}C) acetyl Co A (specific activity 50 mC/mmole) and unlabeled acetyl Co A were added to distilled water in quantities sufficient to obtain a final molarity of 10 mM with a specific activity of 10 mC/mmole. After the substrate was prepared, a spectrophotometric analysis was made at 260 mμ, utilizing the Zeiss spectrophotometer. This was done to ascertain the correct molarity. To accomplish this, 2 μl of the prepared substrate was pipetted into a test tube and 1.0 ml of 0.1 N HCl was added and the contents were mixed well. The sample optical density was then read at 260 mμ, using a molar extinction coefficient of 14.3×10^6 cm^2/mole.* The molar extinction coefficient at 260 mμ of 14.3×10^6 cm^2/mole was found to correspond best to gravimetric values, and all corrections in the final molarity of the substrate were based upon this analysis. However, the variations between calculated molarity and spectrophotometric determination were minimal.

B. Preparation of the Buffer-Substrate Solution.

To prepare the buffer-substrate solution (950 μl), 500 μl of stock solution containing 0.1% bovine serum albumin, 3.80 mM $MgCl_2$, 9.50 mM choline chloride, and 570 mM NaCl were added to 400 μl of 200 mM Na_2HPO_4-NaH_2PO_4 buffer, pH 7.10. To these combined solutions were added 20 μl of 10 mM physostigmine sulphate and 30 μl of the (^{14}C) acetyl Co A substrate solution. The resulting final concentrations were 300 μM acetyl Co A, 5 mM choline chloride, 0.2 mM physostigmine sulphate, 300 mM NaCl, 2 mM $MgCl_2$, 0.05% bovine serum albumin, and 70 mM Na_2HPO_4-NaH_2PO_4. This final solution was stored at -20°C.

C. Preparation of a Solubilized Enzyme Fraction Utilizing Triton X-100.

A convenient source of enzyme was obtained from rat (Sprague-Dawley) whole brain homogenates (20% w/v) after all parts caudal to the superior colliculi were discarded. Before homogenizing, Triton X-100 (0.5%) was added to insure complete solubilization of the enzyme. This concentration of Tritton X-100 released all enzyme and prevented adsorption of the enzyme to dissociated membrane material. The homogenation was then carried out in 0.32 M sucrose using a teflon to glass homogenizing vessel. All procedures were performed at 0° to 2°C. A further dilution of the homogenate was made in 0.32 M sucrose in order to obtain a final homogenate of which a 2-μl aliquot yielded 8 to 10 μg of protein. This was usually a 50% dilution when utilizing rat brain homogenates as the enzyme source.

* Goldberg. Unpublished results.

D. Assay Procedure for the Determination of Choline Acetyltransferase Activity.

The final buffer-substrate solution was prepared immediately before assaying and was then placed in an ice bath (0° to 2°C). Sodium tetraphenylboron/3-heptanone (75 mg/ml) was also prepared and cooled to 0° to 2°C. Since sodium tetraphenylboron/3-heptanone was not stable, new solutions were prepared before each assay. Into chilled microcentrifuge tubes were added 20 µl of buffer-substrate solution and 2 µl rat brain homogenate (8 to 10 µg protein). The solution was mixed well by vortexing. The buffer-substrate with homogenate was incubated at 38°C for 30 minutes. The reaction was stopped by replacing the microcentrifuge tubes into the ice bath. Immediately 150 µl of the sodium tetraphenylboron/3-heptanone solution was added to each tube and mixed well. The solution was centrifuged at 600 x g for 5 minutes. After centrifuging, 100 µl of the organic phase was carefully removed and pipetted into a liquid scintillation vial. Bray's cocktail (17 ml) was added to each vial.

The aliquot in Bray's cocktail was then counted in a Tricarb Model 3375 liquid scintillation spectrometer. Blank determinations were made by replacing the enzyme with water.

The preparations and methods for the handling of micro-quantities of material, as described by Lowry *et al.*,[7] were used throughout our investigations.

E. Protein Determination.

Protein determinations were made by the method of Lowry *et al.*[8] Aliquots were taken for protein determinations before and after the addition of Triton X-100. Those aliquots which were taken after the addition of Triton X-100 were centrifuged at 600 x g for 5 minutes to remove the precipitate caused by Triton X-100 contamination. The optical densities were read at 500 mµ.

IV. RESULTS.

A. Solubilization of Choline Acetyltransferase.

1. The Effects of pH and Ionic Strength.

In solubilizing choline acetyltransferase, adsorption and readsorption become factors when the pH is lower than 7.0 and the ionic strength is lower than that obtained with 300 mM sodium chloride. In accordance with these observations, at pH 7.1 with 300 mM NaCl, we obtained 80% to 90% solubilization of the enzyme with no apparent adsorption or readsorption. This was the maximum value for solubilization which could be obtained utilizing pH and ionic factors (figure 1). Choline acetyltransferase is generally accepted as a soluble enzyme; however, its adsorption properties are such that if the appropriate pH and ionic values are not maintained, the enzyme is readily readsorbed.

[7] Lowry, O., Roberts, N., Leiner, K., Wu, M., and Farr, A. J. Biol. Chem. 207, 1 (1955).

[8] Lowry, O., Rosebrough, N., Farr, A., and Randall, R. J. Biol. Chem. 193, 265 (1951).

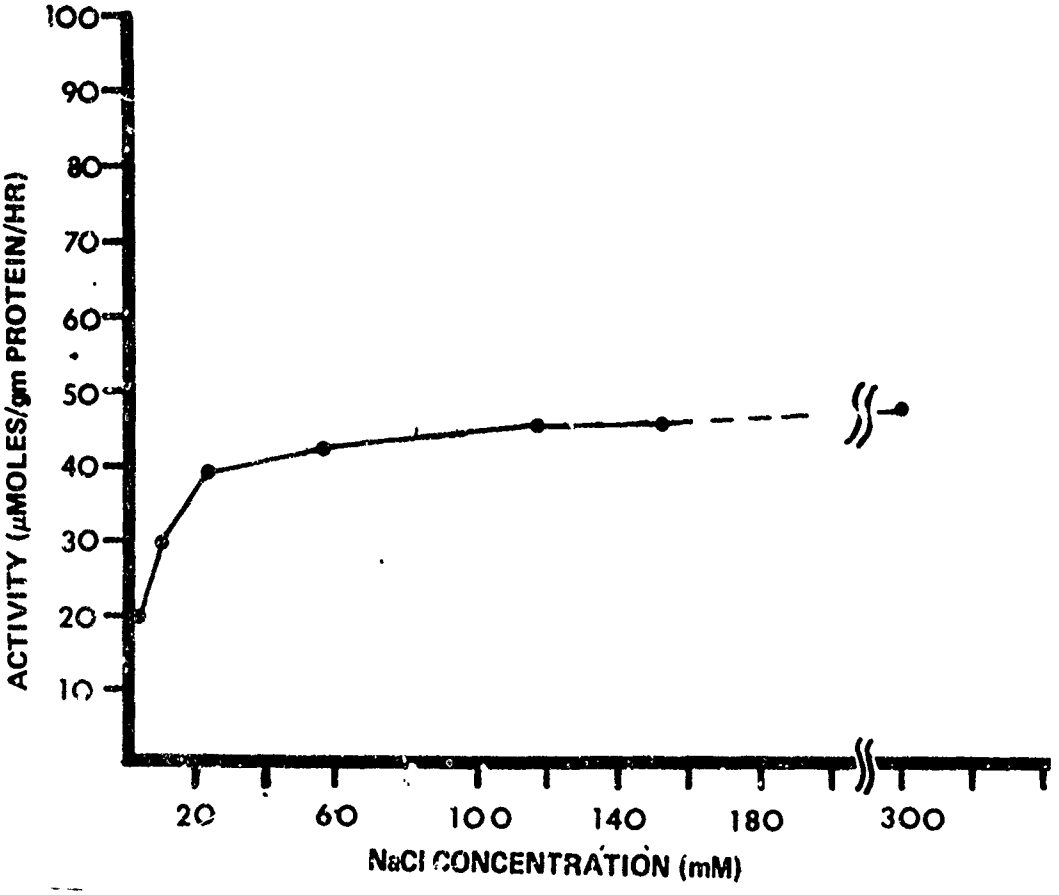

Figure 1. Effects of Sodium Concentration on Choline Acetyltransferase Activity

2. Activation by Triton X-100.

The use of Triton X-100 in initial homogenates resulted in an increase in enzyme activity of 10% to 15%. This increase in enzyme activity may be referred to as activation or as the 100% level of enzyme solubilization. Present evidence suggests that choline acetyltransferase does exist in a membrane-bound form. Therefore, the increase in enzyme activity obtained with Triton X-100 treatment may be due to the release of occluded and membrane-bound enzyme. Thus, in the present enzyme system, adsorption and readsorption of the enzyme to particulate matter are prevented by maintaining a pH of 7.1 and an ionic strength comparable to a final NaCl concentration of 300 mM, whereas Triton X-100 (0.5%) insures release of all membrane-bound enzyme. The enzyme activity obtained with the procedure presented here is considered to be the maximal activity of the crude homogenate or the 100% level of solubilization (table I).

Table I. Effects of Triton X-100 on the Solubilization of Choline Acetyltransferase in Rat Brain

Treatment	μμmoles Acetylcholine synthesized/hour	% Solubilization*
—— 0.32 M Sucrose	181.8	20.0
pH 7.1, 0.32 M Sucrose	310.0	34.6
pH 7.1, 300 mM NaCl	734.0	81.5
pH 7.1, 300 mM NaCl 0.5% Triton X-100	904.0	100.00

* % Solubilization based on solubilization with 0.5% Triton X-100 (100%).

B. Choline Acetyltransferase Activity: Effects of Time, Protein, and Maximal Substrate Concentrations.

The formation of (^{14}C) acetylcholine was proportional to protein concentration over at least a tenfold range. This range, as determined for rat brain tissue, was from 2 to 20 μg of protein (figure 2). The acetylation of choline proceeded linearly for approximately 45 minutes with protein concentrations varying from 2 to 20 μg of protein (figure 3). Determinations were usually made using 8 to 10 μg of protein. When the above conditions were observed, consistent and reproducible results were readily obtained.

The concentration of acetyl Co A necessary to yield maximal activity has been reported to be approximately 0.5 mM.[4] The final concentration of acetyl Co A used in conducting our assays was 0.30 mM. With the use of a substrate concentration approaching the molarity which would yield maximal activity, we were also able to maintain consistently low blank values.

The optimal concentration of choline was found to be 5.0 mM, and changes in choline concentrations ranging from 5.0 to 15 mM did not change the apparent activity.

C. Determination of an Appropriate Blanking System.

The following blank systems were studied in order to determine an appropriate blanking system.

1. Buffer-substrate without tissue
2. Buffer-substrate plus tissue without choline
3. Buffer-substrate with tissue which had been boiled for 30 minutes

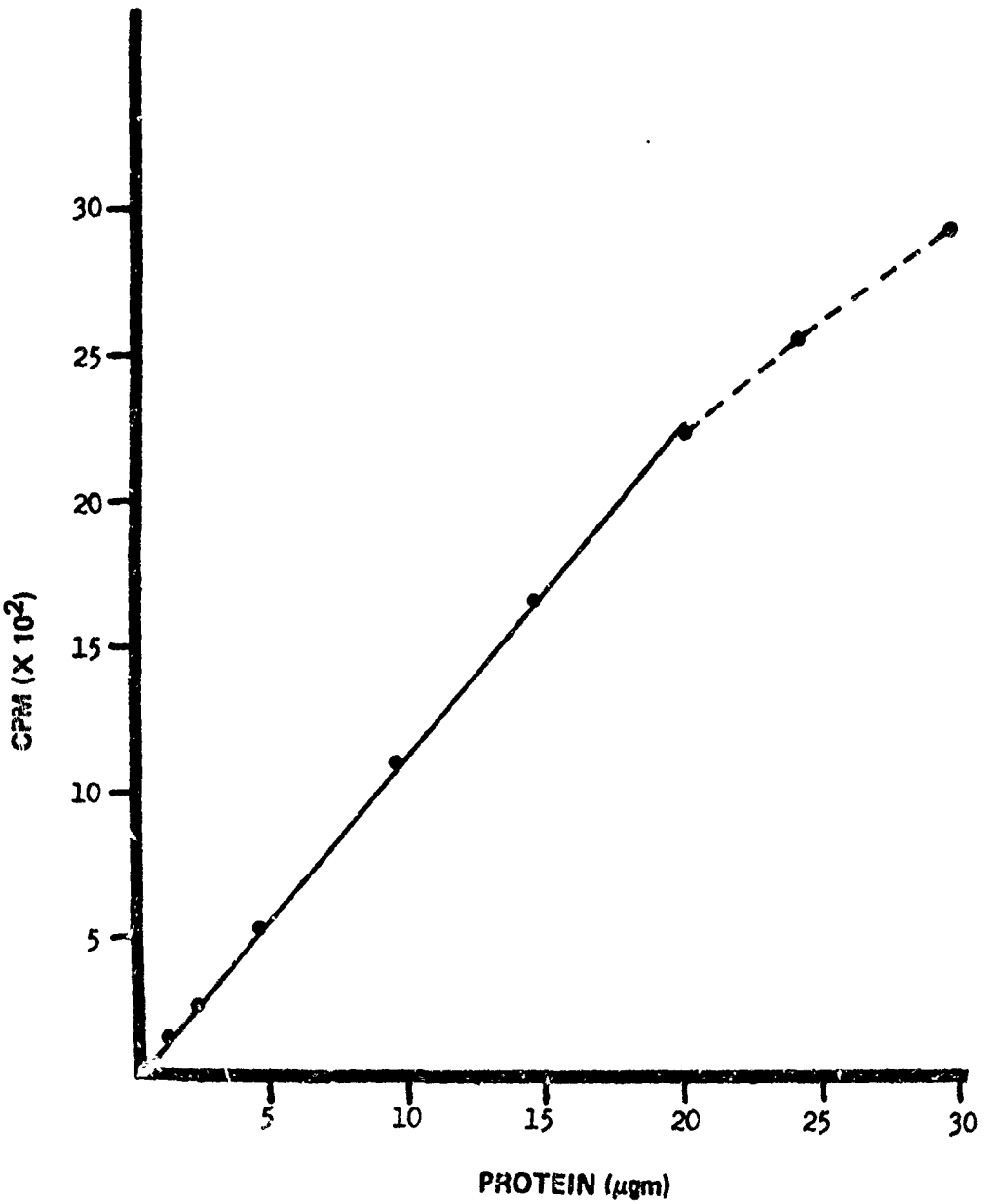

Figure 2. Linearity as a Function of Protein Concentration

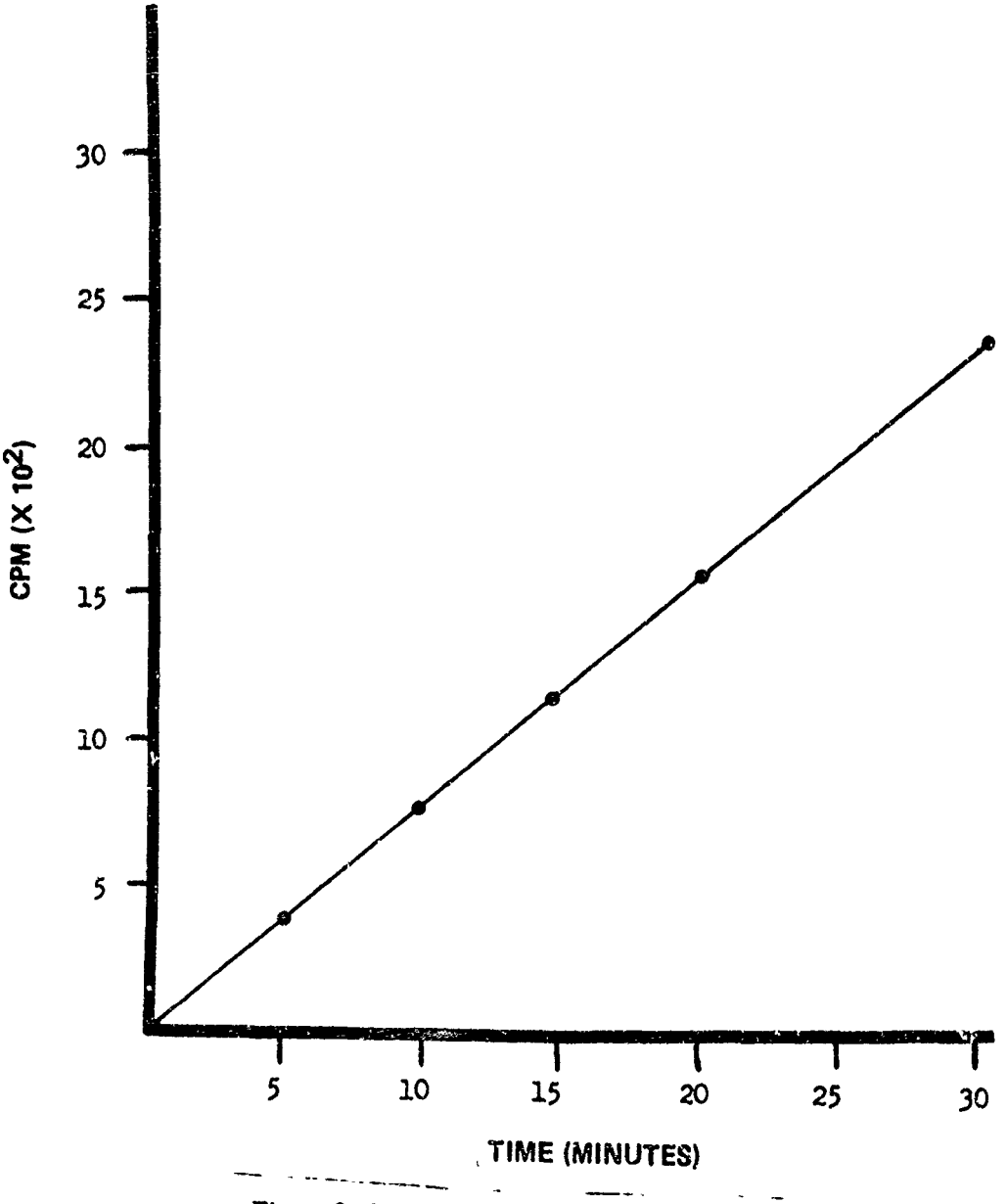

Figure 3. Linearity as a Function of Time

There was no apparent difference in the values obtained for the three blanking systems. For convenience, buffer-substrate without tissue was used as the standard blank for the system (table II).

Table II. Blank Determinations for the Choline Acetyltransferase System

System	Cpm
Buffer-substrate without tissue	254 n = 10
Buffer-substrate plus tissue without choline	288 n = 10
Buffer-substrate with tissue, boiled 30 minutes	265 n = 10

D. Inhibition of Choline Acetyltransferase Activity.

1. Criteria for Inhibition and the Effects of Various Inhibitors.

The inhibition of choline acetyltransferase, if it is to be effective, should meet three criteria:

a. The inhibitor should be highly potent (I_{50}10 μM)

b. It should be capable of passing through the blood-brain barrier and penetrating other membranes

c. It should have a very low affinity for the enzyme, acetylcholinesterase[9]

Since the above criteria are difficult to meet, our inhibition studies at the present time are confined to *in vitro* preparations only. This greatly facilitated the study of the enzyme and the action of its inhibitor. For convenience, a cross section of various drugs and their inhibitory effects on choline acetyltransferase and acetylcholinesterase are given in table III. From this table it can be seen that only two drugs produce an inhibitory effect on choline acetyltransferase. It is known that sulfhydryl group inhibitors have an inhibitory effect on choline acetyltransferase. As expected, the sulfhydryl group inhibitor iodoacetamide produced substantial inhibition. However, iodoacetamide also inhibited acetylcholinesterase; for this reason it is difficult to study in cholinergic systems. The styrylpyridine analogue, 4-(1-naphthylvinyl)-pyridine, produced marked inhibition of choline acetyltransferase but exhibited no inhibitory properties with regards to acetylcholinesterase. Thus, 4-(1-naphthylvinyl)-pyridine is a very effective choline acetyltransferase

[9] Baker, B., and Gibson, R. J. of Med. Chem. 14, 315 (1571).

inhibitor. At the concentrations studied, Soman was a relatively ineffective choline acetyltransferase inhibitor.

Table III. Effects of Various Inhibitors on Choline Acetyltransferase and Acetylcholinesterase Activity

Drug	Concentration	Acetylcholinesterase % inhibition*	Choline acetyltransferase % inhibition**
	mM		
Neostigmine	1.0	100.0	12.5
Soman	0.1	100.0	0
Sernyl	1.0	100.0	0
Hemicholinium-3	1.0	90.0	0
Guanethadine	1.0	87.7	6.4
Carbamyl choline	1.0	85.3	21.8
Bretylium	1.0	84.2	7.3
Atropine sulfate	1.0	79.0	6.5
Chlorpromazine	0.1	56.7	0
Iodoacetamide	0.1	36.5	60.0
Bulboacapnine	1.0	17.5	33.8
2-Mercaptobenzinilamide	1.0	17.0	0
4-(1-Naphthylvinyl)-pyridine	0.1	0	90.0

* Acetyl-1-C^{14} β-methylcholine (substrate)
** Acetyl-1-C^{14} Coenzyme A (substrate)

2. Applications in the Control of Acetylcholine Synthesis and Purification of Choline Acetyltransferase by Affinity Chromatography.

With the advent of specific choline acetyltransferase inhibitors, such as 4-(1-naphthylvinyl)-pyridine, it has become possible to study the regulation of acetylcholine synthesis with greater specificity. The applications of this drug are currently being investigated in our laboratory.

Purification of choline acetyltransferase by affinity chromatography was studied utilizing a meta-carboxyphenyltrimethylammonium Sepharose column with a linear gradient

choline elution. This column is relatively specific for acetylcholinesterase. The column showed no affinity for the enzyme choline acetyltransferase; however, the application of affinity chromatography in columns utilizing specific reversible inhibitors of choline acetyltransferase was not investigated.

V. DISCUSSION.

Methods for the determination of choline acetyltransferase have been described;[4,5,10-14] however, these methods were found to be more meticulous and time consuming than the method we have presented. Moreover, the use of maximal substrate concentrations was prohibited due to the large blank values which were obtained using extraction procedures requiring reinecke or periodide precipitation.

The solubilization of choline acetyltransferase utilizing Triton X-100, as described by Fonnum,[6] resulted in efficient and reproducible results. This procedure also yielded a 100% solubilized enzyme fraction from which the total choline acetyltransferase levels could be determined without hindrance caused by adsorption and readsorption effects. As reported by Fonnum[6] and Potter and Glover,[14] the effects of adsorption and readsorption can be eliminated by maintaining appropriate pH values (7.1 to 7.4) in the presence of sodium chloride (300 mM). These effects occur because the enzyme is more cationic than most proteins. Using the methods of Fonnum[6] we were able to obtain 100% solubilization of the enzyme. The relative specific activity of the enzyme was 60 μmoles acetylcholine synthesized/gram protein/hour.

The determination of the specific activity (60 μmoles/gram protein/hour) presented here correspond with those values reported by Goldberg *et al.*[12] and McCaman and Hunt.[4] The study of various inhibitors of choline acetyltransferase yielded one very effective inhibitor, 4-(1-naphthylvinyl)-pyridine. The inhibition of choline acetyltransferase by styrylpyridine analogues has also been reported by Hemsworth and Foldes.[15] The styrylpyridine inhibitors and various other inhibitors of choline acetyltransferase are now being studied in our laboratories for their potential as regulators of acetylcholine synthesis. The application of affinity chromatography in the purification of acetylcholinesterase was previously reported.* This application resulted in a 1000-fold increase in acetylcholinesterase activity. However, the use of the same ligand in the purification of choline acetyltransferase did not increase the activity of the enzyme. Thus, the meta-carboxyphenyltrimethylammonium Sepharose column exhibits marked specificity for the enzyme acetylcholinesterase. Specific choline acetyltransferase inhibitors and their binding to various column materials are being considered.

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[10] Buckley, G., Consolo, S., Giacobini, E., and McCaman, R. Acta Physiol. Scan. 71,341 (1967).

[11] Fonnum, F. Biochem. J. 115, 465 (1969).

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[15] Hemsworth, B., and Foldes, F. Eur. J. of Pharm. 11, 187 (1970).

* Yamamura et al. In press.

A modification of the choline acetyltransferase assay has been used by Schuberth, Sparf, and Sundwall[16] to approximate acetylcholine turnover rates.

Thus, the determination of choline acetyltransferase activity has many applications with regards to the study of cholinergic systems.

VI. CONCLUSION.

The determination of choline acetyltransferase was facilitated by using a modified extraction procedure (sodium tetraphenylboron/3-heptanone) with which maximum substrate concentrations could be used thus yielding maximum specific activities. Utilizing Triton X-100, a 100% solubilized fraction was obtained and subsequent determination of the activity yielded a relative specific activity of 60 μmoles/gram protein/hour. As a result of these modifications we were able to obtain an efficient and simplified method (sensitivity 10^{-12} moles/hour) for the determination of the cholinergic indicator, choline acetyltransferase.

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[16] Schuberth, J., Sparf, B., and Sundwall, A. J. of Neurochem. 16, 695 (1969).

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